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Inducible expression of a cloned heat shock fusion gene in sea urchin embryos

(microinjection/gene regulation)

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ABSTRACT **A fusion gene construct, in which the coding sequence for bacterial chloramphenicol acetyltransferase (CAT; acetyl-CoA: chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) was placed under the control of the regulatory region of the *Drosophila* gene encoding the 70-kilodalton heat shock protein [Di Nocera, P. P. & Dawid, I. B. (1983) *Proc. Natl. Acad. Sci. USA* 80, 7095–7098], was microinjected into the cytoplasm of unfertilized sea urchin eggs. Pluteus-stage embryos developing from the injected eggs were exposed to high temperature conditions that we found would elicit an endogenous sea urchin heat shock response. These embryos express the gene for CAT and, after heat treatment, display 8–10 times more CAT enzyme activity than do extracts from control embryos cultured at normal temperatures. The injected DNA is present in high molecular weight concatenates and, during development, is amplified about 100-fold. Amplified sequences are responsible for all or most of the induced CAT enzyme activity.**

We recently developed procedures for the microinjection of cloned DNA into sea urchin eggs and determined the fate of the exogenous DNA during development (1, 2). In the present paper, we provide initial evidence that DNA sequences introduced into these eggs can be transcribed and productively expressed, in response to internal physiological signals. The DNA is injected into the cytoplasm before fertilization, sperm is then added, and development is allowed to proceed. We found earlier that when linear DNA molecules are injected, they are rapidly ligated into high molecular weight end-to-end concatenates, and that these undergo extensive replication during the first 24 hr of embryogenesis. The average net amplification of exogenous DNA mass during this period is 25-fold (1); in experiments reported below, an amplification of about 100-fold was observed.

The present experiments were conducted with a fusion gene assembled by Di Nocera and Dawid (3), in which the bacterial structural gene for chloramphenicol acetyltransferase (CAT; acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) is placed under the control of the 5′ regulatory region of the gene coding for the *Drosophila* 70-kDa heat shock protein (hsp70). Di Nocera and Dawid showed that when this construct is transfected into *Drosophila* tissue culture cells, synthesis of the CAT enzyme is induced by heat treatment. Nucleotide sequences that are shared between the several different *Drosophila* heat shock genes have been demonstrated to be required for transcriptional induction under high temperature conditions (4–7). Most important for our present considerations is the finding that the *Drosophila* hsp70 regulatory region functions inductively in heterospecific systems, including the *Xenopus* oocyte nucleus (7, 8), where it is inactive at 20–27°C and induced at 34–37°C; in monkey cells (5, 6) and mouse cells (9), where it is inactive at 37°C and induced at 43°C and 45°C, respectively; and in yeast cells (10), where it is inactive at 24°C and induced at 40°C. In *Drosophila* and *Drosophila* tissue culture cells, the gene is inactive at 25°C and induced at 37°C (3, 11–13). Thus, in transfection experiments the gene seems to respond to whatever temperature is stressful for the cell in which it is placed.

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MATERIALS AND METHODS

Microinjection of Plasmid hsp-cat1 into Unfertilized Eggs. Collection of gametes and microinjection of sea urchin eggs were as developed by McMahon *et al.* (1). About 2 pl of a solution (24 μg/ml) of the plasmid hsp-cat1 (3) dissolved in 40% glycerol was injected into the egg cytoplasm. The plasmid, which is about 5.7 kilobases (kb) in length, was first linearized by digestion at its unique *Bgl* I site, which is located in the pBR322 component of the construct. The amount nominally injected per egg represents about 7500 molecules. As described by Di Nocera and Dawid (3), hsp-cat1 contains a 1.2-kb 5′ regulatory sequence from the *Drosophila* gene for hsp70, cloned by them into pSVO-cat, a vector that contains the Tn*9* structural gene for CAT and simian virus 40 (SV40) early region intron splice and poly(A) addition sequences, but which lacks the early region promoter.

Protein Labeling of Pluteus-Stage Embryos. At 72 hr postfertilization, embryos were suspended at a concentration of 20,000 per ml in 1 ml of Millipore-filtered sea water (FSW). Heat-shocked embryos were incubated for 1, 2, and 4 hr at 25°C, and control embryos were incubated for 4 hr at 15°C in FSW. For protein labeling, the embryos were further incubated for 1 hr at 25°C (heat shocked) or 15°C (control) in the presence of 180 μCi of [^{35}S]methionine (Amersham, >600 Ci/mmol; 1 Ci = 37 GBq). The embryos were pelleted in a Microfuge, washed once in FSW, pelleted again, and suspended in 200 μl of electrophoresis sample buffer containing 0.1 M Cleland's reagent, 2.0% $NaDodSO_4$, 80 mM Tris (pH 6.8), 10% glycerol, and 0.025% bromophenol blue.

Protein Gel Electrophoresis. Aliquots of 20 μl of labeled embryo samples were heated to 80°C for 1 min, spun briefly in a Microfuge to remove debris, and loaded onto a 10% polyacrylamide/$NaDodSO_4$ slab gel. Electrophoresis was as described by Hubbard and Lazarides (14). Gels were soaked in EN^{3}HANCE (New England Nuclear), dried under vacuum, and exposed to Kodak XAR5 film at −70°C for 90 min.

CAT Assay. Heat-shocked or control plutei were pelleted in a Microfuge and suspended in 0.25 M Tris·HCl (pH 7.8). CAT assays were essentially as described by Gorman *et al.* (15) with the following minor alterations. Embryos were lysed by two freeze-thaw cycles in a 95% ethanol/dry ice bath and spun briefly to remove cellular debris. Final reac-

Abbreviations: hsp, heat shock protein; hsp70, 70-kDa hsp; SV40, simian virus 40; CAT, bacterial chloramphenicol acetyltransferase; *cat*, gene for CAT; FSW, Millipore-filtered sea water; kb, kilobase(s).

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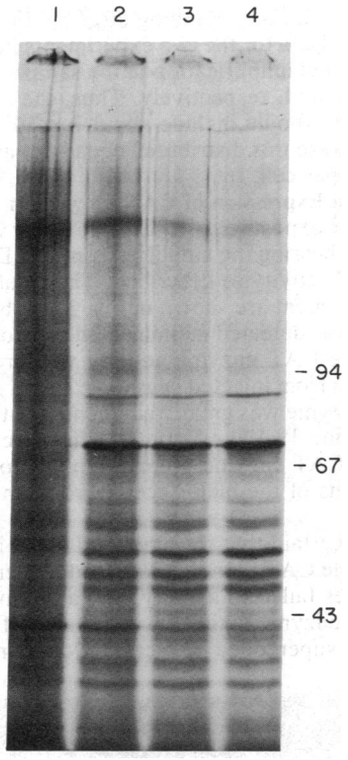

FIG. 1. Heat shock proteins in *S. purpuratus*. Proteins synthesized in pluteus-stage embryos were displayed by gel electrophoresis and autoradiographed. Embryos were maintained at a concentration of 2×10^4 per ml at the normal culture temperature of 15°C for 4 hr (lane 1) or at 25°C for 1 hr (lane 2), 2 hr (lane 3), and 4 hr (lane 4), followed in each case by a further 1 hr of incubation at 15°C or 25°C, respectively, in the presence of [^{35}S]methionine. Size markers are shown in kDa.

tion volumes were 150 μl. This contained 0.5 μCi of [^{14}C]chloramphenicol (45 mCi/mmol, Amersham) and 0.53 mM acetyl CoA (Sigma). Bacterial CAT (Pharmacia P-L Biochemicals) was used as a positive control in CAT assays and in calibrating the reaction. Assays were run for 2 hr at 37°C, and the acetylated products were separated on an Eastman Kodak silica gel TLC plate. TLCs were exposed to Kodak XAR5 film at −70°C.

RESULTS

Heat Shock Response in *Strongylocentrotus purpuratus*. Our initial problem was to determine the optimal heat shock conditions for *S. purpuratus*, which in the laboratory are raised routinely at 15°C. We chose 25°C as an appropriate temperature for studies of protein synthesis after heat shock, on the basis of preliminary survival experiments and unpublished protein synthesis data obtained by B. Brandhorst (personal communication) on the same species. Fig. 1 displays protein synthesis patterns in embryos exposed to 25°C for 2, 3, and 5 hr and in control embryos held at 15°C. Newly synthesized proteins were labeled in each sample by addition of [^{35}S]methionine for the final 1 hr of incubation. As observed in many previous studies, the autoradiograph of newly synthesized proteins of control embryos (15°C; Fig. 1, lane 1) reveals a nearly continuous size distribution. Exposure to 25°C results in a general reduction in protein synthesis and the appearance of several abundant new proteins (lanes 2–4). As in many other organisms (10), the major heat-induced species was about 70 kDa. In addition, a peptide of about 90 kDa and about five lower molecular weight bands that cannot be distinguished in the control sample also were labeled in heat-shocked *S. purpuratus* embryos. Several other fairly abundant proteins appeared to be synthesized in both control and heat-shocked *S. purpuratus* embryos. Fig. 1 shows that the accumulation of the heat shock proteins was already maximal within 2 hr (lane 2) and further incubation (lanes 3 and 4) had little effect. An incubation of 2 hr at 25°C was utilized for the subsequent experiments.

Ligation and Replication During Development of Injected hsp-cat1 DNA. Unfertilized eggs were injected from a continuously flowing microneedle with ≈7500 molecules of hsp-cat1 DNA as described in *Materials and Methods*. The injected hsp-cat1 DNA underwent the expected ligation and replication process (1, 2). This is demonstrated in the autoradiographs shown in Fig. 2. DNA was extracted from pluteus-stage (72 hr) embryos raised from injected eggs as described by McMahon *et al.* (1), run on a 0.8% agarose gel, and after transfer to nitrocellulose, hybridized to the ^{32}P-labeled hsp-cat1 probe. Lanes 1–4 of Fig. 2*a* contain serial dilutions of

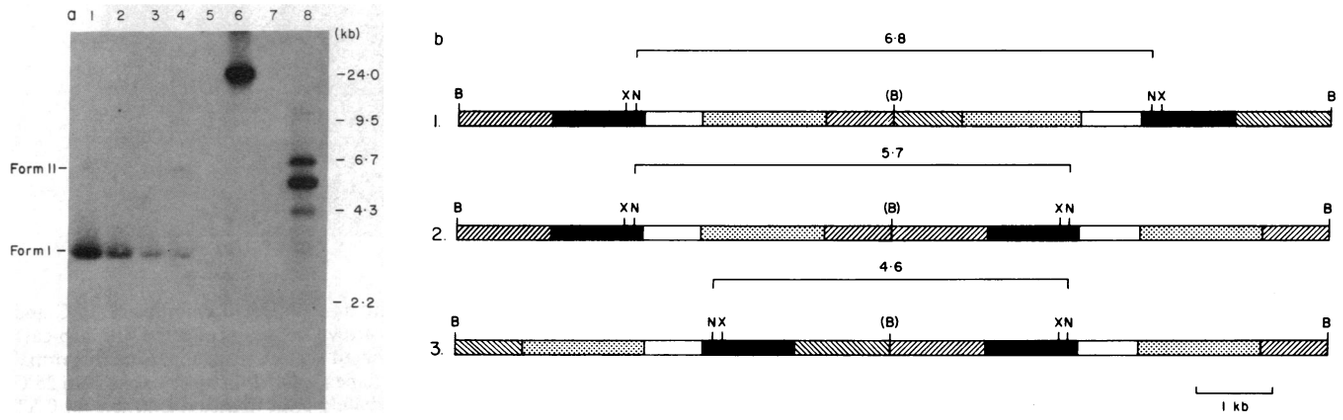

FIG. 2. Concatenation and amplification of injected hsp-cat1 DNA. (*a*) Autoradiograph of gel blot-hybridization between hsp-cat1 probe and DNA extracted from pluteus-stage embryos. Lanes: 1–4, DNA standards containing 1.80×10^7 (lane 1), 0.90×10^7 (lane 2), 0.45×10^7 (lane 3), and 0.22×10^7 (lane 4) molecules of hsp-cat1; 5–8, DNA samples from pluteus-stage embryos: DNA from embryos grown from uninjected eggs (lanes 5 and 7), DNA from embryos derived from eggs injected with hsp-cat1 (lanes 6 and 8), undigested DNA (lanes 5 and 6), and *Nru* I-digested DNA (lanes 7 and 8). DNA size markers (kb) are indicated. Exposure was for 3 hr with an intensifying screen. (*b*) Restriction maps of hsp-cat1 dimers that would be formed by ligation of linearized plasmid at *Bgl* I site. Solid bar, 5′ regulatory region from a *Drosophila* 87C hsp70 gene; open bar, gene for CAT; stippled bar, SV40 "t" intron and early region poly(A) addition sequences; hatched bar, pBR322 [from Di Nocera and Dawid (3)]. Only relevant restriction sites are shown: B, *Bgl* I; X, *Xho* I; N, *Nru* I. Maps: 1, "head to head" concatenate; 2, "head-to-tail" or "tail-to-head" concatenate; 3, "tail-to-tail" concatenate. Sizes of fragments that would be released on *Nru* I digestion are indicated by brackets.

Table 1. Amplification of hsp-cat1 and CAT enzyme activity in pluteus-stage embryos

Data	Exp. 1	Exp. 2
Amplification of hsp-cat1*	×107	×99
CAT enzyme activity		
Control embryos (15°C)		
Acetylation of [^{14}C]CA†	0.9%	0.8%
Units of CAT activity per sample†	7.2×10^{-4}	6.9×10^{-4}
Heat-shocked embryos (25°C)		
Acetylation of [^{14}C]CA†	7.5%	8.0%
Units of CAT activity per sample†	29.5×10^{-4}	31.2×10^{-4}
Estimated molecules of CAT subunit per embryo‡	0.91×10^{6}	0.90×10^{6}
Molec. CAT subunit§ / molec. hsp-cat1	0.86	0.85

*Amplification of injected sequences was estimated from densitometry or scintillation counting of pooled embryo DNA samples with reference to known standards as in Fig. 2. Each egg was assumed to have received 2 pl of a solution (24.0 μg/ml) of hsp-cat1, or 7.5×10^3 molecules.

†Percentage acetylation of chloramphenicol (CA) was determined by scintillation counting of substrate and product spots after TLC. Corresponding CAT enzyme activity was then determined by reference to the standard curve, using the equations given in the legend to Fig. 4.

‡Estimates of the number of CAT enzyme molecules were made by utilizing a nearly pure CAT enzyme preparation as a specific activity standard. The CAT enzyme preparation was produced in tissue culture using a baculovirus expression vector as described by Smith *et al.* (24). In $NaDodSO_4$ gels, the only protein visible in this preparation is the 25.6-kDa CAT subunit (15). The specific activity, S, measured as in Fig. 4, was about 90 units/μg of protein (this, of course, could be an underestimate because some of the enzyme molecules in the standard preparation could have been inactive). The number of enzyme subunit molecules per embryo is 2.35×10^{13} A/SN, where 2.35×10^{13} is the number of 25.6-kDa subunits per μg, A is the measured number of units of activity per sample, and N is the number of embryos in the sample, about 840, for Exp. 1 and 900 for Exp. 2. The estimates given here and in the bottom row assume that all embryos contain injected, amplified hsp-cat sequences. However, Flytzanis *et al.* (2) have shown that an average of only 60% of larvae actually contain amplified exogenous DNA after injection. Thus positive larvae could easily have expressed as much as 2-fold more molecules of CAT enzyme than shown.

§(‡ row)/(* row) (7.5×10^3), where 7.5×10^3 is the number of hsp-cat1 molecules injected per egg.

hsp-cat1 as reference standards. No hybridization of the probe was detected with DNA from uninjected embryos (lanes 5 and 7), whereas in the undigested DNA sample from injected eggs (lane 6), the probe hybridized to a high molecular weight band that occupied the same position as the total high molecular weight genomic DNA of the embryos. On digestion with *Nru* I, three reactive fragments of approximately 6.8, 5.7, and 4.6 kb were observed. These are exactly the fragments predicted if the injected plasmid forms a random concatenate by end-to-end ligation (1), as illustrated in Fig. 2*b*. Thus, with arbitrary reference to the *Bgl* I end closest to the SV40 sequences in linearized hsp-cat1 as the "head" and the other end as the "tail," digestion of a random end-to-end concatenate with *Nru* I would generate the reactive head-to-head (Fig. 2*b*, map 1), head-to-tail (Fig. 2*b*, map 2), and tail-to-tail (Fig. 2*b*, map 3) fragments of the lengths shown.

Reference to the DNA standards included in Fig. 2*a* indicates that the mass of the exogenous DNA was amplified greatly during development. Had no amplification occurred, with total recovery assumed, only about 3.4×10^5 molecules of hsp-cat1, or 2.2 pg, would have been present in the 45 embryos included in the experiment. This is less by a factor of 6.5 than the DNA content of the lowest standard, in lane 4 of Fig. 2*a*. The extent of amplification was estimated by densitometry or scintillation counting after hybridization to the hsp-cat1 probe. Data for the two experiments included in Table 1 indicate a net amplification in the sample as a whole of 99-fold and 107-fold, respectively. Thus, the average pluteus-stage embryo should include about 7×10^5 molecules of hsp-cat1 and, were this distributed equally to all cells, about 500 molecules per cell.

Heat-Induced Expression of CAT Enzyme in Pluteus-Stage Embryos. After exposure to 15°C or 25°C for 2 hr, pluteus-stage embryos bearing the amplified hsp-cat1 DNA were assayed for CAT activity as described. The results of a representative experiment are shown in Fig. 3. No background of CAT activity was detected in uninjected control plutei (lanes 2 and 4). Some CAT enzyme activity was present in 15°C embryos grown from injected eggs (lane 1) and the amount of active CAT enzyme was greatly augmented in the heat-treated embryos (lane 3). Lane 5 contains the reaction products of a bacterial CAT enzyme preparation to provide markers for the positions of the major acetylated forms of chloramphenicol.

It is almost certain that the amplified DNA is responsible for the inducible CAT activity. As noted earlier, supercoiled DNA molecules failed to amplify, and a relatively very low level of CAT enzyme activity was obtained from embryos injected with supercoiled hsp-cat1. Occasionally, we en-

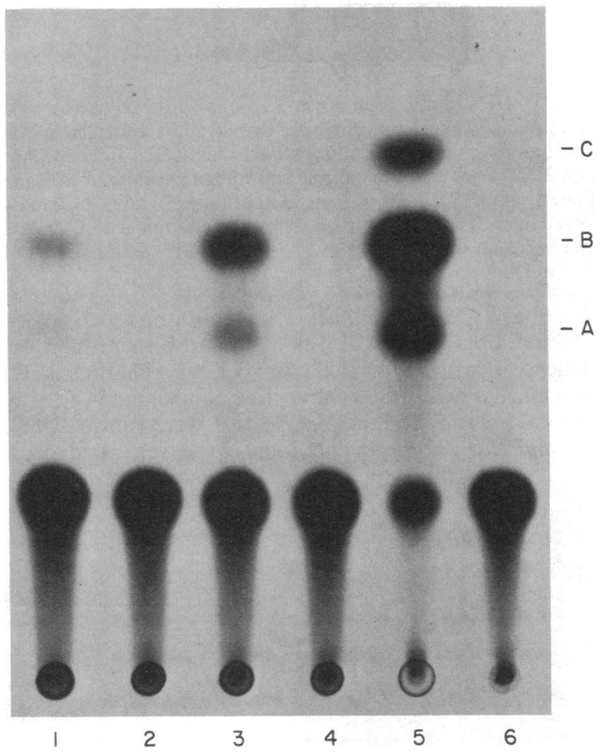

FIG. 3. CAT enzyme activity in injected embryos at 15°C and 25°C. Pluteus-stage embryos grown from eggs injected with hsp-cat1 and control plutei from uninjected eggs were incubated at the normal culture temperature of 15°C (lanes 1 and 2) or were exposed to 25°C for 2 hr (lanes 3 and 4) immediately prior to assay. Extracts for CAT assay were prepared from 837 and 836 injected plutei (lanes 1 and 3, respectively) and 1000 control plutei (lanes 2 and 4). Bacterial CAT enzyme (0.37 units) (lane 5) and the assay reaction mixture with no enzyme or extract added (lane 6) were assayed in parallel as described. Acetylated reaction products were separated from unacetylated [^{14}C]chloramphenicol substrate by ascending TLC. Reaction products (A, B, and C) are indicated. The primary and predominant product of the reaction is 3-acetyl chloramphenicol (B). Other products are a diacetylated form (C) and a product of an inefficient, nonenzymatic acyl migration (A) (16, 17). Autoradiography was for 24 hr.

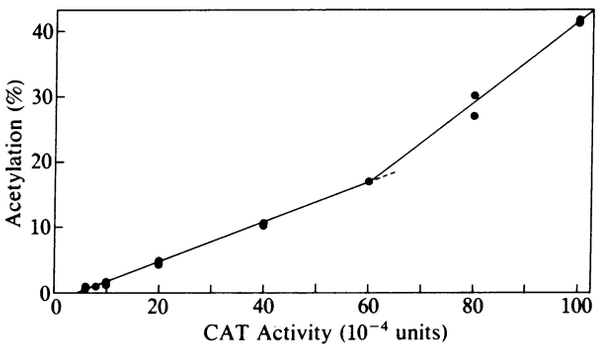

FIG. 4. Assay of CAT enzyme activity. Enzyme activity was measured in 2-hr reactions, as described by Gorman *et al.* (16) and in *Materials and Methods*. Commercial bacterial CAT enzyme was utilized in the presence of L cell extracts. The specific activity (0.37 units/μl) of the starting preparation was calibrated by the manufacturer. A unit is defined as the amount sufficient to acetylate 1 μmol of chloramphenicol per min (18). The abscissa is calibrated in 10^{-4} units, termed "U" in the following data. At U < 60, by linear least squares, A = 0.297U − 1.25, where A is % acetylation. At U = 60, the second acetylation product first becomes visible in autoradiographs of CAT assay carried out as in Fig. 3. At this point the slope doubles. Where U = 60–100, the solution is A = 0.619U − 20.5. The small abscissa intercept in the initial phase of the curve shown (A = 0 at U = 4.2) indicates that there is a slight inhibition of CAT activity caused by unknown substances in the cellular extracts. At the specific activity of the [^{14}C]chloramphenicol utilized, the sensitivity of the assay is about 5000 cpm per % acetylated products formed, though of course this value varies according to the length of time the reaction is allowed to proceed, the radioactivity of precursor utilized, and other such factors.

counter batches of eggs that fail to ligate and amplify injected linear DNA, although the DNA remains intact (1). This occurred in an experiment carried out with hsp-cat1 DNA (data not shown). Exogenous DNA could just be detected in the pluteus-stage embryos of this experiment, and the amount of CAT activity was less than 1% of that measured in the experiment shown in Fig. 3. Were the induced expression of CAT the product of a small subfraction of the hsp-cat1 molecules that do not undergo amplification, the amount of enzyme activity recovered would be expected to be independent of the net DNA amplification, contrary to the result obtained.

Approximate Quantities of CAT Enzyme Synthesized in Heat-Shocked Embryos. An easily detectable signal was obtained from about 840 injected, heat-treated embryos (Fig. 3). The sample chromatographed in lane 3 contained about 4 × 10^4 cpm of [^{14}C]acetylated chloramphenicol. To prepare the number of pluteus-stage embryos included in the heat-treated and control samples together (i.e., lanes 1 and 3) required 3 days of microinjection. Thus, the extremely high sensitivity of the CAT assay (16) offers for these purposes an important practical advantage. In control studies carried out with cultures of L cells transformed with a construct bearing the same CAT gene, we compared directly the signal obtained by S1 nuclease measurement of CAT mRNA and by CAT enzyme assay. Using a high-specific-activity single-stranded probe generated from an M13 primer, we recovered a protected fragment of the appropriate size containing about 1 cpm from a number of L cells (10^6) that yielded sufficient CAT activity to produce about 10^5 cpm of [^{14}C]acetylated chloramphenicol products. This result indicated that, for monitoring CAT gene expression in practical numbers of injected embryos, the enzyme assay would be greatly preferable.

The fraction of chloramphenicol acetylated in the assay was linear with enzyme activity over the range of these experiments (Fig. 4). The molecular size of the purified enzyme is known (15), and from its specific activity, the number of molecules synthesized in the heat-induced sea urchin embryos can be calculated. Data for two experiments are given in Table 1. It can be seen that the extracts from heat-induced embryos produced 8- to 10-fold more acetylated chloramphenicol than did the extracts from the 15°C embryos, which (because of the abscissa intercept of the standard curve) translates to 4–5 times more units of enzyme activity. The calculated number of molecules of CAT enzyme subunit after induction is about 10^6 per embryo. Table 1 also shows that, were all of the amplified hsp-cat1 DNA sequences active, the average (steady state) yield in heat-treated embryos would be on the order of 1 enzyme subunit molecule per gene copy. Since all may not be active and since all embryos may not contain amplified hsp-cat1 DNA (see the legend to Table 1), these are to be regarded as minimal estimates.

DISCUSSION

These results show that exogenous DNA sequences injected into the cytoplasm of the unfertilized sea urchin egg can be productively expressed during embryological development. The injected DNA also undergoes massive net amplification during development, as noted for a number of other plasmid sequences (1). Though there is as yet no direct cytological evidence, the observations that at least some of the exogenous sequences participate in both transcription and replication imply strongly that they have been sequestered into the embryo nuclei. Furthermore, the amplified hsp-cat1 sequences in the embryos are at least to some extent inducible. Several consequences follow from the observation that the *Drosophila* hsp70 control sequences can be activated in heat-stressed sea urchin embryos. First, new evidence is provided to the effect that the heat shock gene functions at the distress temperature of the host cells, here only 25°C, though in the species of origin 25°C is the standard environmental culture condition at which the gene is silent. Second, these results imply that at least some of the exogenous DNA present in the pluteus-stage embryos exists in a physical conformation such that sequence-specific regulatory interactions (4, 5, 7) may take place. Third, induction occurs in the presence of a number of hsp-cat1 molecules that is probably, on the average, 50–100 times the number of endogenous heat shock gene sequences. This implies a sufficient excess of diffusible regulatory molecules so that at least some of the hsp70 control sequences are engaged at least some of the time. It would be interesting to determine whether the presence of the injected hsp70 sequences affects the transcription of the endogenous heat shock genes.

The great sensitivity of the CAT assay (see the legend to Fig. 4) means that the signal obtained even after heat induction could be the product of fairly rare mRNAs. This is indeed suggested by calculation of the number of mRNA molecules required to produce the estimated number of CAT enzyme subunits in 2 hr. On the assumption that the rate of CAT mRNA translation at 25°C is equal to the rate of translation of sea urchin histone mRNA at 15°C, which has been measured at 0.7 codons per sec (19), to accumulate 10^6 CAT subunit molecules in 2 hr would require about 5000 RNAs per embryo, or four molecules per average cell. Of course, were translation of this heterologous sequence several times less efficient, as observed for the *Drosophila* hsp70 gene in *Xenopus* oocytes (7), proportionately more mRNAs would be needed. Nonetheless, this result implies that the induced CAT mRNAs are far less prevalent than those evidently required for the synthesis of the prominent endogenous heat shock proteins in the same embryos (see Fig. 1). The probable low ratio of CAT mRNAs per total hsp-cat1 sequence (Table 1) means either that only a small fraction of the exogenous genes participate at any one time in the induction or

that the CAT mRNA itself is unstable, or perhaps both. It may be relevant that only 0.1–0.2% of L cells receiving nuclear microinjections of pBR322 containing a thymidine kinase gene stably express this gene after a few rounds of DNA replication (20).

There are many possible reasons why the observed ratio of induced-to-uninduced CAT activities is not higher than 8–10 (Table 1). In *Drosophila* tissue culture cells transformed with hsp-cat1, a ratio of about 30 in CAT activity was obtained (3). A larger fraction of the amplified hsp-cat1 molecules in sea urchin embryos may be competent to express than to express inductively, or the concentration of positive regulators may be insufficient. Furthermore, the *Drosophila* mRNA hsp70 leader sequence in fact may not be recognized in sea urchin cells in such a way as to permit efficient translation at high temperature (7). A major factor may be the extent of expression in uninduced (15°C) embryos. Higher uninduced levels of expression of the gene for hsp70 than expected have been reported in several other transfection experiments (3, 21, 22). We could possibly have stimulated a mild heat shock response in the control embryos by maltreating them during the collection and centrifugation process. Finally, it is possible that the rate of expression at 15°C is indeed very low, but that the enzyme is stable, and thus that we are comparing the induced enzyme synthesized in 2 hr at 25°C to the uninduced enzyme accumulated over the preceding 3 days. Whatever the explanation, it remains that expression of some of the exogenous genes for CAT is clearly activated under the correct physiological conditions.

We do not yet know whether sea urchin genes that are ontogenically regulated and that normally function only in certain embryonic cell lineages will perform with fidelity when introduced into unfertilized eggs in the same manner. We have so far demonstrated merely that environmentally modulated expression can occur from some fraction of the exogenous genes. However, it is important to explore the possibility that ontogenic regulation can be studied by these methods as well. The sea urchin embryo offers a unique and important advantage for the study of embryological development—the availability of many cloned genes that operate in specific early cell lineages (23). The nature of the *cis* acting genomic information required for the embryonic regulation of these genes may be accessible by the simple and practical experimental route described in this initial study.

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